Extended data

Material and Methods

Histology.
In situ hybridization and immunochemistry have been described in[1]. Diaphorase staining on cryostat sections was performed as described in ref[2].
Immunofluorescence on cryostat or vibratome sections was performed as previously described[3]. Whole-processed embryos where fixed overnight in 4% paraformaldehyde (in PBS) and dissected spinal cords where fixed for 2 hours at room temperature. Antigen retrieval, by boiling for 10 minutes in sodium citrate (10mM) was needed for optimal labeling with the α-Islet antibody.

Wholemount immunofluorescent staining using the 3DISCO method was adapted from ref [4]. All steps up to the imaging of the embryos were performed under nutation. Embryos at stage E11.5 were fixed overnight in 4% paraformaldehyde (in PBS), serially dehydrated in graded methanol (in PBS) up to 100% methanol and then bleached using Dent's bleach overnight at 4°C. Following serial washes in 100% methanol, the embryos were incubated in Dent's fixative overnight at 4°C. The embryos were then serially rehydrated in graded methanol (in PBS) up until re-immersion in PBS. Embryos were further subjected to incubation at 70°C to optimize antigen recognition by the anti-Phox2b antibody. Following washes in PBS-Tween (0.1%), tiny superficial perforations were made in the embryo with a minutien pin to facilitate antibody penetration. The embryos were then incubated with primary antibodies in blocking buffer (20% DMSO, 5% FCS in PBS) for 5 days at room temperature. Following washes in PBS-Tween (0.1%) at room temperature, secondary antibodies in blocking buffer were then applied for 4 days at room temperature. Finally, embryos were cleared following the 3DISCO protocol subsequent to washes in PBS-Tween (0.1%) at room temperature, Embryos were imaged using a SP8 confocal microscope (Leica). 3D reconstructions and videos were obtained using the IMARIS imaging software.

Antibodies
The following primary antibodies were used for immunochemistry and immunofluorescent staining:

α-2H3 (NF), Mouse, 1:500, Hybridoma Bank (#2H3)
α-bIII Tubulin (Tuj1), Mouse, 1:500, Covance (#MMS-435P)
α-dsRed, Rabbit, 1:500, Clontech (#632496)
α-Tomato, Goat, 1:1000, Sicgen (#AB0040-200)
α-Islet1:2, Mouse, 1:400 (40.2D6 and 39.4D5, Hybridoma Bank)
α-Phox2b, Rabbit, 1:500[5]
α-Phox2b, Guinea Pig, 1:500[6]
α-Phox2a, Rabbit, 1:500[7]
α-Sox10, Goat, 1:250, Santa Cruz (#SC-17342)
α-FoxP1, Rabbit, 1:200 (#AB-16645)

The following secondary antibodies were used:

α-rabbit Cy3, 1:500, Jackson Immunoresearch Laboratories (#711-165-152)
α-rabbit A488, 1:500, Jackson Immunoresearch Laboratories (#711-545-152)
α-goat Cy3, 1:500, Jackson Immunoresearch Laboratories (#705-166-147)
α-goat A647, 1:500, Jackson Immunoresearch Laboratories (#705-606-147)
α-rabbit Cy3, 1:500, Jackson Immunoresearch Laboratories (#711-165-152)
α-mouse Cy3, 1:500, Jackson Immunoresearch Laboratories (#715-165-150)
α-mouse A488, 1:500, Invitrogen (#A-21202)
α-mouse Cy5, 1:500, Jackson Immunoresearch Laboratories (#715-175-150)

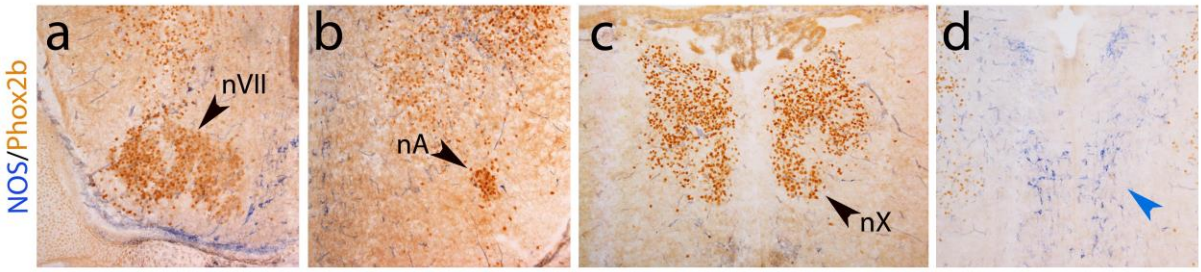
Immunohistochemical reactions were processed with the Vectastain Elite ABC kits (PK-6101 and PK-6012; Vector Laboratories) as per manufacturer's guidelines followed by colour development using DAB (3,3'-Diaminobenzidine).

Probes
For the *Phox2b* riboprobe, primers containing SP6 and T7 overhangs were used to amplify a 635 bp region (nucleotides 123 – 757) from a plasmid containing the full-length *Phox2b* cDNA sequence. The purified amplicon was then used as the template for antisense probe synthesis using T7 RNA polymerase.
Forward Primer: 5'-CCGTCTCCACATCCATCTTT-3'
Reverse Primer: 5'-TCAGTGCTCTTGGCCTCTTT-3'
The other probes were: *Gata3* (gift of JD Engel), *Hand1* (Stratagene), *Hmx2* (gift of E.E. Turner), *Hmx3* (gift of S. Mansour), *Islet1*, *Tbx2* (gift of A. Kispert), *Tbx3* (gift of V.M Christoffels),Tbx20[8], VAChT (Source BioScience, UK, 40129421 (CK3-a14) IMAGE clone).

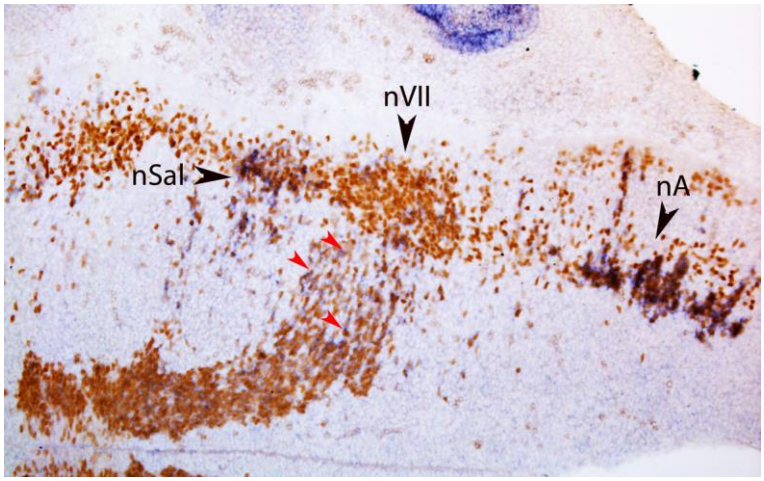
Transgenic Mouse Lines:
-*Phox2b::Cre* [9]: BAC transgenic line expressing Cre under the control of the Phox2b promoter.
-Rosalox-stop-lox-tdTomato (*RosatdT*) [10]: Knock in line expressing the reporter gene tdTomato from the Rosa locus in a Cre-dependent manner.
-*Phox2b*$^{LacZ/+}$ [11]: Knock in line expressing the reporter gene LacZ from the second exon of the *Phox2b* locus, which is disrupted and lead to a null phenotype in Phox2bLacZ/LacZ embryos.
-*Olig2*^{Cre} line[12]: Knock in of *Cre* in the *Olig2* locus.

All animal studies were done in accordance with the guidelines issued by the French Ministry of Agriculture and have been approved by the Direction Départementale des Services Vétérinaires de Paris.

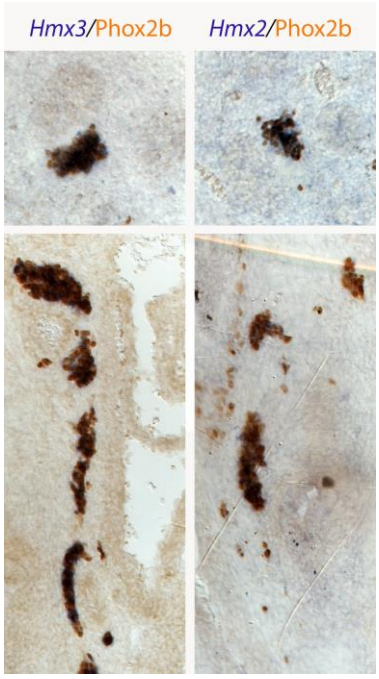
Pixel Classification, Segmentation and Image Analyses.
To measure the size of the pelvic ganglion on cryosections from E13.5 *Olig2*$^{Cre/+}$ and *Olig2*$^{Cre/Cre}$ embryos hybridized for *Phox2b* and immunostained for neurofilament, we used the open source image analyses tool ilastik[13]. Pixels were segmented by a Random Forest Classifier into signal (corresponding to the pelvic ganglion) and background (corresponding to surrounding tissues and nerve fibers). Segmentation on one section was optimized through an iterative training procedure based on color/intensity, edge and texture, and subsequently applied to the batch processing of all sections passing through one pelvic ganglion. Local neighborhoods for calculating edge and texture were defined as 3 X 3 pixels & 5 X 5 pixels. Finally, scattered signal areas smaller than 0.2µm² were removed *on FIJI.* The remaining signal area corresponded to the pelvic ganglion and was measured on 5 to 6 consecutive sections, depending on ganglia. The volume of the ganglion was deduced by multiplying the surface by the thickness of the sections, 20µm. Wild-type and mutant ganglia were compared by a paired two-tailed Student t-test.



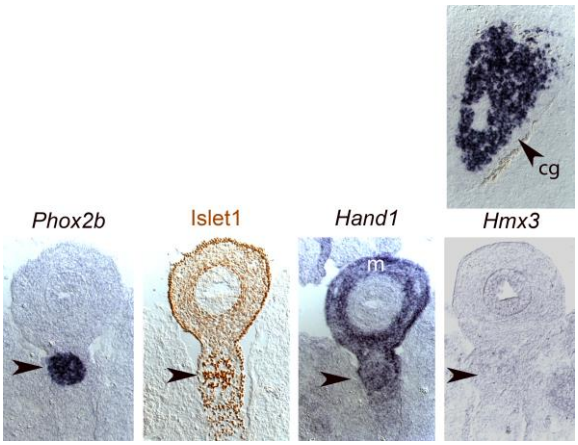
Extended data figure 1
NOS is not expressed in branchiomotor or hindbrain visceromotor neurons. Transverse sections of the hindbrain at E17.5 stained for diaphorase activity and Phox2b immunohistochemisty and passing through: **a**, the facial nucleus (nVII); **b**: the nucleus ambiguous (nA); **c**, the dorsal nucleus of the vagus nerve (nX); **d**, the pons, showing NOS+ neurons of the raphe (blue arrowhead). No double Phox2b^{+}/NOS^{+} neurons were found in the hindbrain.



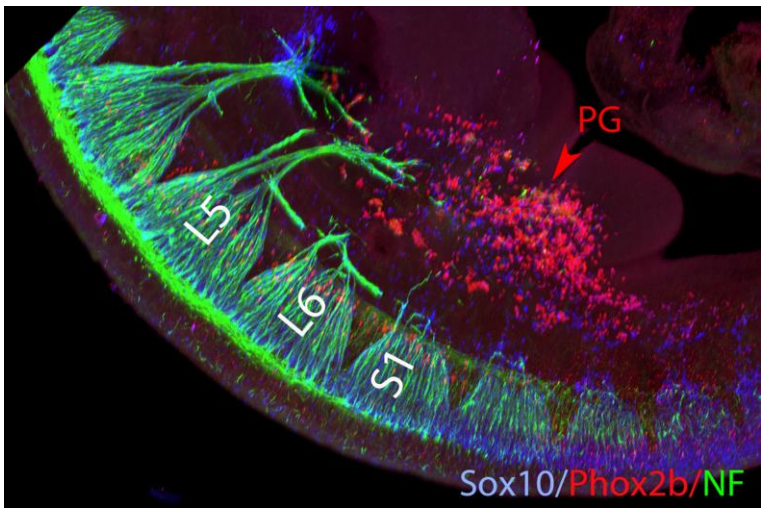
Extended data figure 2
Expression of *Tbx3* in all branchial and visceral motoneurons of the hindbrain. Longitudinal section though an E11.5 medulla, stained by combined Phox2b immunohostochemistry and Tbx3 in situ hybridization. In addition to nX (figure 2), Tbx3 is expressed in salivatory motoneurons (nSal) and the nucleus ambiguus (nA). Expression is also found in a subset of migrating facial motoneuronal precursors (red arrowheads). nVII: facial motor nucleus.



Extended data figure 3
Expression of *Hmx2* and *Hmx3* in cardiac and ciliary ganglia. Parasagittal sections in an E13.5 embryo stained for immunhistochemistry against Phox2b and *Hmx3* (left) or *Hmx2* (right) in situ hybridization, showing expression of the latter genes in the ciliary ganglion (upper panels) and the cardiac ganglia (lower panels).



Extended data figure 4
The ganglion of Remak has a sympathetic identity.
Transverse sections through a chicken embryo at 5 days post fertilization, passing through the hindgut. The ganglion of Remak (arrowhead) coexpresses Phox2b with the sympathetic markers *Islet* (detected by an Islet1-2 antibody) and *Hand1*, but not the parasympathetic marker *Hmx3*, which is expressed at the same stage in the ciliary ganglion (cg). Islet and *Hand1* are also expressed in the mesenchymal wall of the gut (m).



Extended data figure 5
The pelvic ganglion forms in the absence of the pelvic nerve
Wholemount immunofluorescence with the indicated antibodies on an Olig2$^{-/-}$ littermate of the E11.5 embryo shown in Fig. 4. Occasionally, no nerve projection is seen at all towards the pelvic ganglion, which nevertheless is present and indistinguishable from its counterpart in wild-type embryos (see Fig. 4). L6: sixth lumbar root. PG: pelvic ganlion.

Extended data figure 6: Schematic of the revised anatomy.

Extended data Movie 1
Movie of the pelvic ganglion at E11.5 in a wild type.

Extended data Movie2
Movie of the pelvic ganglion at E11.5 in an *Olig2* null mutant.

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